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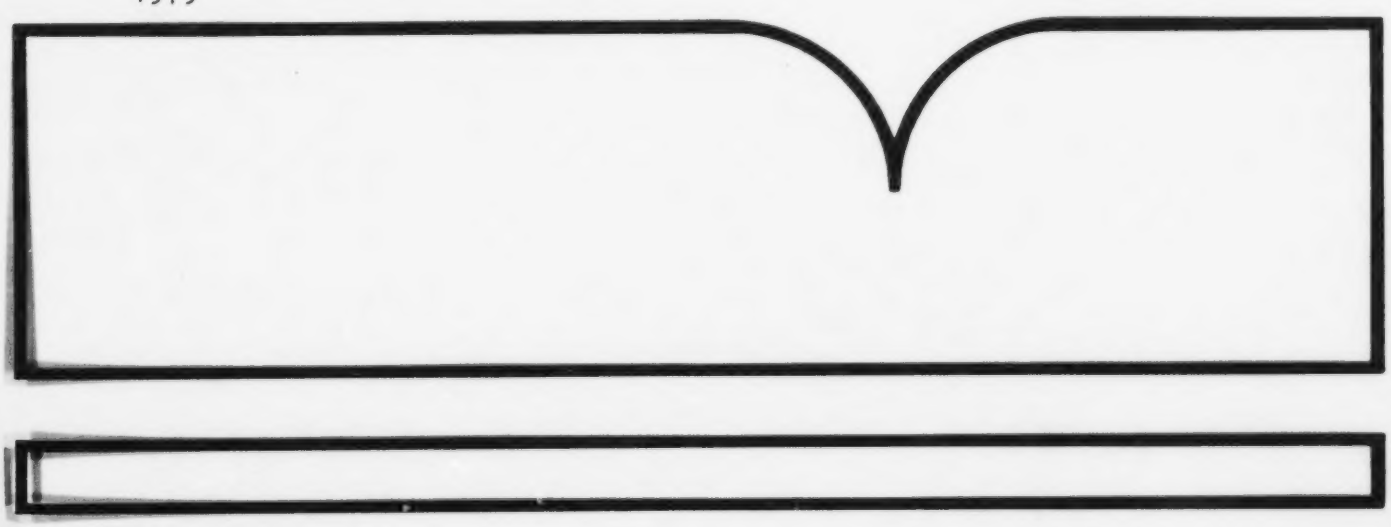

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16. ABSTRACT

The fate of organic pollutants in aquatic ecosystems depends, in part, on metabolic activities of the indigenous microflora. Knowledge is therefore needed for the growth characteristics of aquatic bacteria in low nutrient conditions typical of many aquatic environments. We have studied changes in bacterial populations from Lake Ontario, using continuous culture enrichments, with lactose as the sole carbon and energy source. Enrichment studies have shown that the concn of lactose, the type of water utilized for inflowing media, and the inoculum source affected the outcome of continuous culture enrichments from fresh-water samples. Two isolates from enrichments experiments, an Aeromonas sp. and a Pseudomonas sp., were obtained using concn of 50 and 5 mg/liter lactose, respectively. These isolates were shown to be competitive with each other at different lactose concn. The Pseudomonas sp. was more sensitive to starvation and heat treatment than the Aeromonas sp., and did not take up oxygen in the presence of glucose when cell suspensions were prepared from cultures grown in peptone broth.

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Pollutants Bacteria Aquatic Microbiology	Microflora Continuous culture enrichments Lactose Aeromonas sp. Pseudomonas sp.	08/A 06/F 06/C 06/T

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CHAPTER 55

Characteristics of Bacteria Adapted to Low Nutrient Conditions in Lake Ontario*

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The fate of organic pollutants in aquatic ecosystems depends, in part, on metabolic activities of the indigenous microflora. Knowledge is therefore needed of the growth characteristics of aquatic bacteria in low nutrient conditions typical of many aquatic environments. We have studied changes in bacterial populations from Lake Ontario using continuous culture enrichments, with lactose as the sole carbon and energy source. Enrichment studies have shown that the concn of lactose, the type of water utilized for inflowing media, and the inoculum source affected the outcome of continuous culture enrichments from fresh-water samples. Two isolates from enrichment experiments, an *Aeromonas* sp. and a *Pseudomonas* sp., were obtained using concn of 50 and 5 mg/liter lactose, respectively. These isolates were shown to be competitive with each other at different lactose concn. The *Pseudomonas* sp. was more sensitive to starvation and heat treatment than the *Aeromonas* sp. and did not take up oxygen in the presence of glucose when cell suspensions were prepared from cultures grown in peptone broth.

INTRODUCTION

Natural waters frequently contain very low nutrient concn. Organic materials may be present at concn ranging from 0.1 to 10 mg/liter (Wood 1970; Menzel 1964; Birge and Juday 1934). Dissolved organic compounds originate, in part, from phytoplankton activity (Fogg 1963; Ryther 1963), decay of dead organisms, excretions of zooplankton and larger animals, and leaching from land.

Aquatic environments also receive significant organic loadings from a large variety of agricultural and industrial activities and it is presumed that most of these materials are assimilated into the natural carbon mineralization cycles (Freed et al. 1977). As a result, these environments frequently serve as disposal systems for refractory and nonrefractory wastes such as pesticides, sewage, oil products, and industrial effluents. As a disposal resource, an aquatic environment has a limited capacity to assimilate such wastes, as evidenced with several organochlorine compounds (Edwards 1977). It is important, therefore, to develop the capability to assess load capacity of aquatic environments before the limit is exceeded.

In developing this capability, a more complete understanding of how organic materials are biodegraded at low concn in aquatic environments will be required. Aquatic microbial populations apparently are adapted to grow at low nutrient concn (Jannasch 1967; Ishida and Kadota 1975; Tempest and Neijssel 1976). The bacteria contained therein are referred to as "autochthonous" bacteria (Jannasch 1967). Information on how these bacteria ac-

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commodate and metabolize synthetic organic pollutants, particularly in the presence of natural, readily degradable materials, is sparse.

Similarly, physiological differences between autochthonous and zymogenous (not adapted to grow at low nutrient concn) bacteria have not been studied extensively. Characterization of these differences may greatly aid our understanding of degradation capabilities of autochthonous bacteria. Jannasch (1968) noted that the growth parameters of autochthonous bacterial isolates changed if they were grown for prolonged periods on rich media. The starvation and stress studies of Wu and Klein (1976) delineated major physiological differences between *Escherichia coli* (zymogenous) and indigenous aquatic microorganisms (autochthonous), leading to the exclusion of *E. coli* from natural aquatic environments.

As a prerequisite to understanding the biodegradation mechanisms of natural aquatic microbial populations, it is important to isolate and characterize autochthonous bacteria. Jannasch (1967), Ishida and Kadota (1974), and Kuenen et al. (1977) demonstrated that continuous culture enrichments are an adequate tool for isolating these bacteria. This paper reports results on the physiology and ecology of autochthonous bacteria isolated by the continuous culture enrichment technique.

MATERIALS AND METHODS

Continuous culture enrichments. Continuous culture enrichments were carried out according to Jannasch (1967). The continuous culture system consisted of a 1-liter growth vessel with a 300-ml working volume. Volume was maintained by a positive air pressure overflow. Contents of the growth vessel were mixed by a magnetic stirrer. Reservoir medium was pumped through the continuous culture system using a peristaltic pump (Model 1203 Harvard Apparatus Co.,[1] Millis, MA) and a vapor barrier to prevent back-contamination of the reservoir medium.

Reservoirs were prepared by adding either filter-sterilized (0.22 μm membrane filter, Millipore Corp., Bedford, MA) tap water (diluted 1:4 with distilled water) or filter-sterilized Lake Ontario water to autoclaved 4-liter carboys. The lake water was collected from a dock at Lake Ontario State Park and was boiled for 5 min prior to filter sterilization. Sterile fluids in the reservoirs were then supplemented with 0.6 mM NH_4Cl, 0.05 mM $MgSO_4 \cdot 7H_2O$), and 0.09 mM potassium phosphate buffer (pH 7.2). Depending on the experiment, lactose was added at either 5 or 50 mg/liter. At the 50 mg/liter concn, this gave a C:N:P:Mg ratio of 10:4:1:1 which assured that only the carbon concn would be growth-limiting (Rao and Dutka 1974). Lactose was chosen as the carbon source because future experiments will examine the metabolic regulation of β-galactosidase (a well characterized enzyme) in autochthonous bacteria capable of metabolizing lactose. Although the lowest lactose concn used in these experiments is probably higher than *in situ* concn in lake-water environments (Hutchinson 1975), it was chosen because it gave explicit results; i.e., enrichment was a function of lactose concn rather than a function of an unknown organic material originating from cellular deterioration.

Enrichment experiments were performed by placing a 300-ml sample of fresh Lake Ontario water (collected with sterile bottles along the shoreline of Lake Ontario State Park) in the continuous culture growth vessel and supplementing with lactose, phosphate buffer (pH

[1]Mention of trade names or commercial products does not constitute endorsement by EPA.

7.2), ammonium chloride, and magnesium sulfate at 10 times the reservoir concn to stimulate growth of the mixed population. The vessel was then incubated as a batch culture, with stirring and aeration (filter-sterilized air) for 48 h. Thereafter, flow of the reservoir medium was commenced at a dilution rate (D) = 0.1 h^{-1}. All systems were incubated at room temp, 25 ± 2 C.

Changes in population composition during enrichment experiments were monitored by standard plate count techniques. One-milliliter samples were periodically removed aseptically from the growth vessels, serially diluted in sterile distilled water dilution blanks, and plated by spreading on agar medium (LYP) containing 0.1 g lactose, 0.5 g Bacto peptone (Difco), 0.25 g Bacto yeast extract, and 15 g Bacto agar/liter of distilled water. Colony morphologies on agar plates were distinct enough to permit designation of predominant bacterial types without detailed taxonomic studies of the individual bacteria. Enrichment was considered to have occurred if any colony morphological type reached 60-85% of total colony count on agar plates.

To avoid misleading enrichment results due to growth and adherence of bacteria to the growth vessel walls, experiments were temporarily interrupted (5 min) and the contents of the growth vessel poured into an identical sterile vessel. This new vessel was then sealed with sterile stoppers and tubing as before and the experiment continued. Reservoirs were not changed.

Cultures. The predominant bacterial colony type from each continuous culture enrichment was picked from the agar and streaked once for purity on the LYP medium. Pure cultures were transferred to LYP agar slants and stored at 10 C. Slant cultures were not transferred in order to maintain original physiological characteristics; i.e., cultures continued to give the same growth responses if subsampled from the original slant. Identification of organisms was accomplished using tests cited in Buchanan and Gibbons (1974).

E. coli B was obtained from the culture collection at S.U.N.Y. Brockport, Brockport, NY and maintained, through periodic transfer, on nutrient agar (Difco) slants at 10 C.

Starvation and heat stress. These experiments were modifications of those of Wu and Klein (1976). Cultures were grown in LYP broth for 18 to 48 h on a shaker at 25 C. The cells in 40-ml aliquots of the culture were washed twice (by centrifugation and resuspension) using sterile phosphate buffer (0.31 M, pH 7.2), and then starved by finally resuspending the washed cells in 50 ml of the same phosphate buffer in 250-ml Erlenmeyer flasks (referred to as "starvation flasks"). The flasks were maintained at 25 C on the shaker. Each flask was weighed weekly and evaporated water was replaced by addition of sterile distilled water.

At the beginning of each starvation period and twice weekly thereafter, duplicate 5.0-ml samples were withdrawn from the starvation flasks. One sample was immediately diluted serially and plated to determine the number of viable cells in the starved population. The other sample was subjected to a temp increase of 27 ± 0.5 C (test tube at 52 C for 5 min). The sample was then cooled to 25 C, diluted serially in phosphate buffer, and plated on LYP agar. All dilutions and platings were done in duplicate. Plates were incubated at 25 ± 2 C for 1 wk and decreases in population density determined by standard count techniques.

Oxygen uptake studies. Cultures were grown and harvested as described in the starvation and stress experiment except final suspensions were adjusted to an optical density of 0.2 in a Coleman spectrophotometer (Coleman Industries, Oak Brook, IL) at 550 nm.

Oxygen uptake was determined with a 3-ml cell suspension in a magnetically stirred biological oxygen monitor (Model 53 Yellow Springs Instrument Co., Yellow Springs, OH). After equilibrium, 0.3 ml of substrate solution was added with a hyperdermic needle to the reaction chamber. Oxygen uptake was monitored continuously by a recorder. All uptake studies were repeated at least three times. Statistical differences between oxygen uptake rates were determined by analysis of variance.

RESULTS

Enrichment Studies

Fig. 1 shows the results of four simultaneous continuous culture enrichment experiments using 300-ml aliquots from the same Lake Ontario water sample as the inoculum. Lactose was the sole source of carbon and energy. Predominant colony types were arbitrarily assigned letters based on gross differences in colony morphology. Fig. 1 shows that the appearance of a predominant colony type was a function of both lactose concn and type of water used in the reservoir. With distilled water in the reservoir, colony type J predominated at the 50 mg/liter lactose concn, whereas colony type U predominated at the 5 mg/liter lactose concn. Continued incubation after 12 retention volumes did not alter predominance. Colony types J and U, however, did not predominate when distilled water was replaced with lake water. The predominance of colony types M and P, in this latter experiment, was a function of lactose concn. These results are similar to those reported by Jannasch (1967) in which sea water was used.

The changes in predominant colony types shown in Fig. 1 were typical of many of the continuous culture enrichments we have performed with water samples taken from the same general area along Lake Ontario beaches at different times of the year. As would be expected (Jannasch 1967) with water samples taken on different days, a variety of colony types predominated despite the use of identical experimental protocols. However, the same colony type frequently was observed if water samples taken at different points along the coast on the same day were used as inocula for continuous culture enrichments. Fig. 2

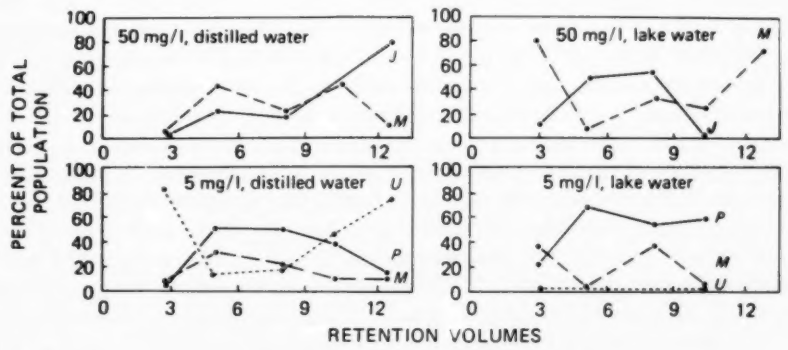


FIG. 1. Predominant colony types (expressed as percentage of total colonies on plates of viable counts) appearing during simultaneous continuous culture enrichments with the same lake-water inoculum, demonstrating the effects of lactose concn (50 or 5 mg/liter) and water type utilized in reservoir media. Letters J, M, P, and U represent different bacterial colony types.

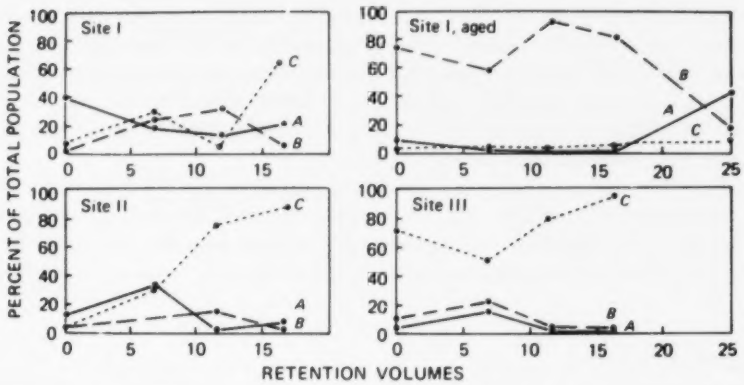


FIG. 2. Predominant colony types (expressed as percentage of total colonies on plates of viable counts) appearing during simultaneous continuous culture enrichments utilizing lake-water inocula from three different sites (I, II, III) along Lake Ontario Shoreline and lactose as sole carbon and energy source. Site I aged refers to the water sample from site I which was stored at 25 C for 48 h. Letters A, B, C represent different bacterial colony types.

depicts the results of an exemplary enrichment experiment of this type. Four simultaneous enrichment experiments were conducted with separate inocula (300-ml water samples) taken on the same day at shoreline sites along Lake Ontario State Park spaced 8.1 km apart. Lactose, at 50 mg/liter in the reservoir medium, was the sole source of carbon and energy. The same colony type C predominated regardless of the inoculum source. These results suggest that ecological niches may exist for certain bacterial types (as defined in this study by colony morphology). Similar results have been obtained at lactose concn of 5 mg/liter. Fig. 2 also shows the effects of aging the water sample (storage at 25 C for 48 h) before using it as inoculum for a continuous culture enrichment experiment. Colony type C, which predominated when unaged water samples were used as inocula, did not predominate after 17 retention volumes when the inoculum was aged.

From one series of experiments performed above, the predominant colony type appearing at the end of two separate continuous culture enrichment experiments, one with 5 and the other with 50 mg/liter lactose in the reservoir medium, was selected for further study. Taxonomic investigation showed one colony type to be a *Pseudomonas* sp. and the other an *Aeromonas* sp., respectively. These isolates were maintained on LYP slants at 10 C, without transfer, to preserve their original physiological characteristics. There was no evidence (slow growth, death, reduced activity, etc.) that these cultures deteriorated over the 12-mo experimental period.

To determine competitive relationships, each isolate was inoculated into separate continuous culture vessels and cultured under the original enrichment conditions. After steady-rate population densities were established, the contents of the two vessels were mixed aseptically. The mixture was divided equally between two sterile continuous culture systems and medium flow resumed at a dilution rate of 0.1 h^{-1}. One mixed culture received reservoir medium at 50 mg/liter lactose and the other received 5 mg/liter. Subsequently, both systems were assayed for changes in population density of each isolate (colony morphologies were readily distinguishable).

Competition between the two isolates as a function of reservoir lactose concn is shown in Fig. 3. At the 5 mg/liter lactose concn, *Pseudomonas* predominated. *Aeromonas* gradually decreased in population density, suggesting that it could not compete successfully with *Pseudomonas* for available lactose. Analysis of the washout rate of *Aeromonas* according to the method of Jannasch (1969) showed it was growing but at a rate slower than *Pseudomonas* and would thus not predominate.

At the 50 mg/liter lactose concn, neither isolate washed out; they coexisted in the continuous culture system for greater than 30 retention volumes. Because of a presumed low substrate saturation constant (Ks), *Pseudomonas* probably subsisted either on the steady concn of lactose, or on some metabolic product produced by *Aeromonas* as it grew on the lactose.

Starvation and Heat Stress

Fig. 4 shows the effects of starvation and heat stress on the two aquatic isolates and *E. coli*; the latter presumably would not be a naturally occurring aquatic inhabitant. These stresses were designed to measure relative sensitivity among the bacteria tested and not to necessarily stimulate a particular environment condition. *Aeromonas* and *E. coli* maintained constant cell densities throughout the starvation period, indicating no substantial cell death. When periodic subsamples of these starved cultures were exposed to a mild heat treatment (see materials and methods), no loss of viability occurred.

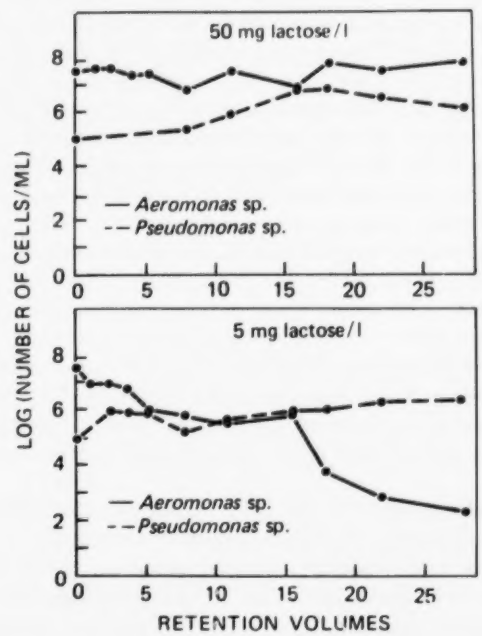


FIG. 3. Effect of lactose concn in the reservoirs on steady state growth of *Aeromonas* sp. and *Pseudomonas* sp. in chemostat culture.

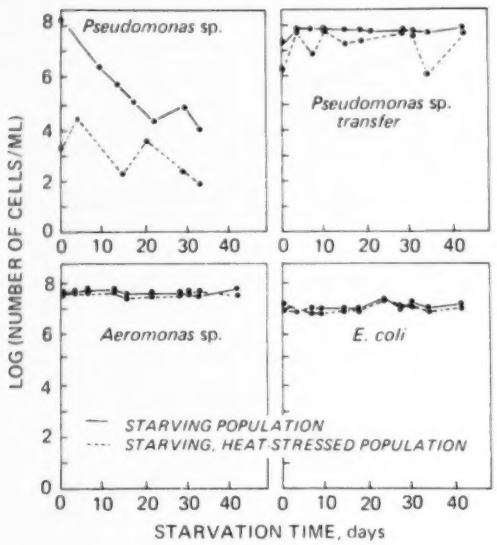


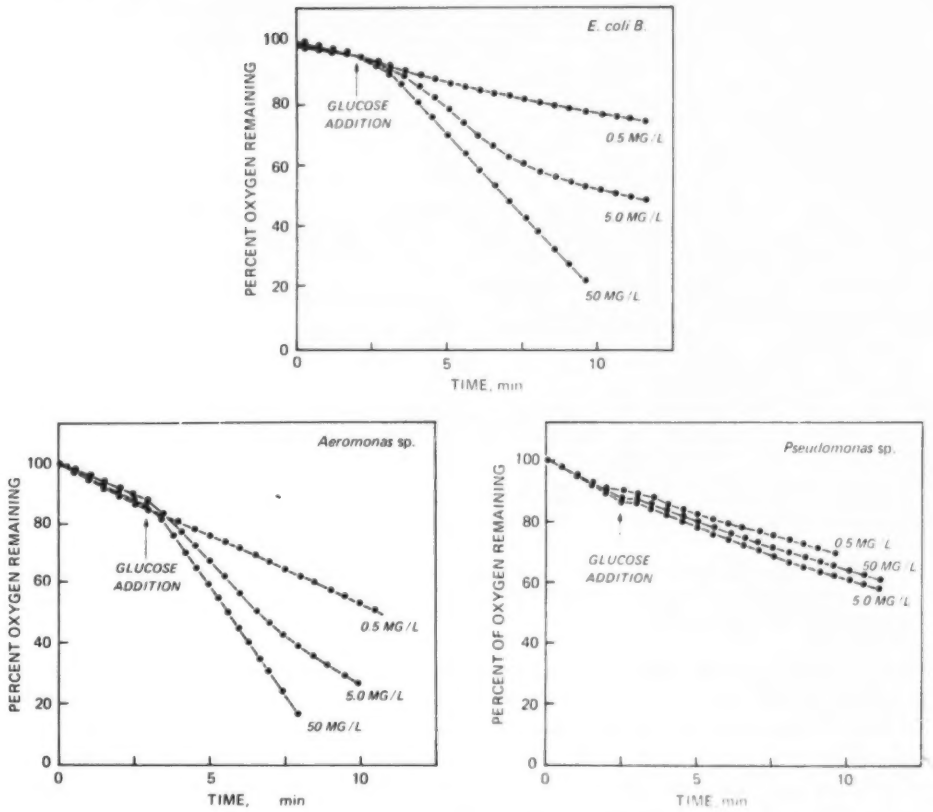
FIG. 4. Total viable cells of *Aeromonas* sp., *Pseudomonas* sp., and *E. coli* B as affected by starvation and heat stress. *Pseudomonas* sp. transfer reflects the same test on a *Pseudomonas* sp. culture after three successive transfers on a rich agar medium.

Pseudomonas, on the other hand, steadily lost viability with continued starvation. Heat treatment further reduced viability; death of starved cells was massive during the earlier periods of starvation, decreasing somewhat thereafter. *Pseudomonas* cultures that were transferred twice successively on the LYP slants became tolerant to starvation and less sensitive to the heat treatment. This rapid physiological change of a culture as a result of transfers on rich media was characteristic only of those isolates enriched at low substrate concn. Decreases in temp or increases in salinity did not affect the viability of starved *Pseudomonas* cells (unpubl. results).

Oxygen Uptake

In their comparative study of effects of starvation on *E. coli* and an aquatic bacterium, Wu and Klein (1976) noted that respiration rates of the two organisms were affected differently after glucose additions. After 3 wk of starvation, their aquatic bacterial isolate showed only a slight increase in oxygen uptake above endogenous levels when glucose was added, whereas *E. coli* showed appreciable oxygen uptake rate subsequent to glucose addition.

To determine whether our isolates would exhibit corresponding differences in oxygen uptake, we grew *Pseudomonas, Aeromonas,* and *E. coli B* in peptone broth. Washed cell suspensions were then prepared and oxygen uptake rates before and after addition of glucose were measured. The results are shown in Figs. 5-7. *Pseudomonas* did not take up oxygen above endogenous levels when glucose was added. The rates of uptake before and after addition of glucose were not significantly different ($\alpha = 0.05$). *Aeromonas* and *E. coli* both took up oxygen at rates significantly ($\alpha = 0.05$) greater than endogenous levels and in proportion to the amount of glucose added.



FIGS. 5-7. Oxygen uptake by *E. coli*, *Aeromonas* sp., and *Pseudomonas* sp. Cells were grown in a peptone medium, washed in 0.31 M phosphate buffer (pH 7.2), and resuspended at an optical density of 0.2 in the same buffer. Mg/liter concn are the amounts of glucose added.

Since these cultures were originally grown in peptone broth, they all showed oxygen uptake when various amino acids were added (not shown). Lactose did not induce oxygen uptake in any of the isolates (within the time course of the experiment) except when the isolates were grown originally on that substrate.

DISCUSSION

Our results with continuous culture enrichments using fresh-water samples parallel those reported by Jannasch (1967) and Ishida and Kadota (1975) for sea-water samples. It is apparent from all of these studies that growth-limiting concn of carbon and energy sources, or some other environmental parameter, determine the type of bacteria which predominant in an enrichment experiment. For example, Kuenen et al. (1977) have shown that different growth-limiting concn of phosphate promote changes in the predominant species seen in continuous culture enrichment experiments.

Very little information exists, however, about how much of a concn difference in carbon and energy source used in a continuous enrichment experiment is required before a change

in the predominant bacterial type occurs and what it means environmentally. Our data indicate a minimum difference of one order of magnitude or less will elicit an enrichment. It is possible that particular aquatic bacteria may be adapted to a specific concn range of a nutrient or environmental parameter, and thus continuous culture enrichment experiments may provide a means of delineating the environmentally controlled degradation potential of aquatic bacteria (that is, a type of ecological niche). Our preliminary data, showing the same predominant colony type in enrichments using the same experimental conditions but inocula collected at different sampling sites along the Lake Ontario shoreline, lend support to this possibility. Ishida and Kadota (1975) have noted specific aquatic bacteria (e.g., *Vibrio* sp.) which consistently predominated in enrichments using unpolluted seawater (DOC: 0.9 mg C/ml) as the growth-limiting factor and inocula from sites in Osaka Bay, Japan.

The appearance of predominant colony types in our continuous culture enrichments was dependent not only on concn of lactose but on the type of water used, that is, the predominant colony type in an enrichment experiment changed when lake water was replaced with distilled water in the reservoir. This would suggest the presence of nutrients or growth factors in lake water which were not available to the bacteria in distilled water. Presumably, the use of a minimal salts reservoir medium prepared with distilled water will select, in a continuous culture enrichment experiment, for the least fastidious members of an aquatic bacterial population. These bacteria will grow most efficiently in oligotrophic fresh-water conditions. The use of minimal salts reservoir medium prepared with lake water will select for a member of an aquatic bacterial population which has a requirement for growth factors other than the principle substrate (lactose in this case) and inorganic nutrients supplied (a fastidious organism). These bacteria will grow most efficiently in less oligotrophic conditions. However, since these trophic conditions are not mutually exclusive, both representative types probably would coexist within many aquatic environments. This would account for their presence in the same water sample as shown in our enrichment experiments.

Physiological characteristics of two aquatic bacteria isolated during our continuous culture enrichment experiments were quite different. The *Pseudomonas* sp. was more sensitive (cell death) to both starvation and mild heat treatment than the *Aeromonas* sp. If it is assumed the continuous culture experiments forced predominant bacterial types to appear based strictly on growth limitation by the lactose, then these different responses to starvation and heat treatment may be a direct function of the lactose concn used in the enrichments. By implication, this suggests that aquatic bacteria capable of efficient growth at low nutrient concn (in our case, *Pseudomonas,* which was the predominate bacterium at 5 mg/liter lactose) may be more sensitive to starvation and/or temp fluctuation in aquatic environments than those capable of growth at high concn.

In a related study, Wu and Klein (1976) obtained an *Aeromonas* sp. from a mountain stream by batch culture enrichment, which lost little viability (ability to grow on agar medium) under starved conditions but there was marked loss of viability when starved cells were heat-treated (53 C, 5 min). The *E. coli* used in their study lost viability under both starvation and heat treatment. Our *Aeromonas* sp. from Lake Ontario and our *E. coli* tolerated (no loss of viability) the same conditions of starvation and heat treatment. Our *Pseudomonas,* on the other hand, which can be considered an autochthonous bacterium (Jannasch 1967), proved to be very sensitive, losing viability rapidly when incubated under either condition. Our results, then, in conjunction with those of Wu and Klein (1976) in-

dicate that sensitivity to starvation and heat treatment is equivocal as an explanation of why *E. coli* (not an autochthonous bacterium in aquatic environments) cannot persist in natural aquatic environments.

The change in response to starvation and heat stress, as noted when *Pseudomonas* was transferred two successive times in a rich medium, is a phenomenon similar to that reported by Jannasch (1968) for sea-water isolates. Caution should be exercised in handling these cultures to insure preservation of original physiological characteristics. We had no problem maintaining *Pseudomonas* and *Aeromonas* on LYP slants at 10 C without transfer. Jannasch (1968) found that sea-water isolates remained physiologically stable in filter-sterilized, unsupplemented seawater, and we found this to be true as well, of our isolates in lake water.

The oxygen uptake responses further demonstrate physiological differences between the two isolates obtained during continuous culture experiments at different lactose concn. When *E. coli* and *Aeromonas* were grown in a peptone medium (amino acids, the only source of carbon and energy), washed cell suspensions readily took up oxygen when given glucose. Cell suspensions of the *Pseudomonas* consistently showed no oxygen uptake in the presence of glucose. A similar observation was reported by Wu and Klein (1976). This oxygen uptake response may reflect a physiological condition unique to the *Pseudomonas* sp. and possibly to other autochthonous aquatic bacteria.

A continued study of the physiology and metabolic capacities of autochthonous bacteria in aquatic environments will hopefully lead to a better understanding of the organic mineralization processes which they directly control. Since many man-mobilized synthetic organic chemicals are entering aquatic environments at low concn, we expect that their transformation and ultimate fate will depend on incorporation into these mineralization processes. The clearer our understanding of the degradative capacities of autochthonous bacteria, the more readily we will be able to predict the fate of synthetic organics in aquatic environments.

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